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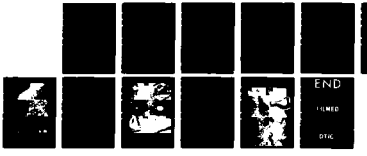

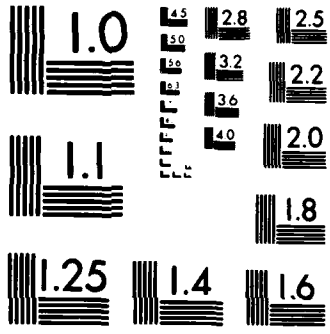

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Tissue Culture and In Vivo Modelling of Corneal Opacification and Ocular Injuries Induced by Millimeter Waves

Annual Summary Report

DR. JOHN R. TREVITHICK

June 1981

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Conditions were established for in vitro culturing of rabbit corneas in preparation for experiments in which irradiation with millimeter waves would be performed in vitro. Alpha medium was found to be appropriate for culturing corneal epithelial cells and whole corneas for short periods up to one week. Incubation of corneas at elevated temperatures from 39°-50°C resulted in progressive disruption of normal cell structure, beginning at low temperatures (39, 42) with apparent swelling of cells, loss of normal structure of microville, and depressed rather than raised cell junctions, and at higher temperatures (43°C,

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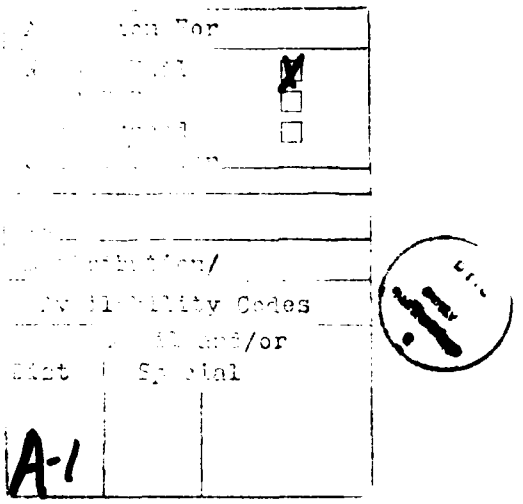
20. Abstract (Con't)

50°C) to loss of microvilli, curling of cells and deepithelialization.



Summary

The eventual aims of these experiments are to use intact corneas incubated in vitro and corneas of rats exposed in vivo to elevated temperatures or high energy pulsed millimeter waves to study the development of corneal damage. Such experiments are expected (1) to establish conditions for corneal damage and (2) to elucidate the mechanisms by which the damage occurs.

If intact corneal explants are cultured in tissue culture dishes in medium at 35.5°C, continuing outgrowth of the epithelial cells occurs from the edge of the explants during a period of at least two weeks. Exposure of such cultured corneals to elevated temperatures followed by fixation for scanning electron microscopy (SEM) revealed progressive cellular damage as the temperature increased. At low temperatures (37°C) the cellular changes were confined to a swollen appearance of the cell border. At higher temperatures, progressive changes including curling up of normally flat cells and eventually denudation of large areas of the cornea occurred. It is hoped that a new technique developed for the visualization of the cytoplasmic contents of cells by removal of the cell membrane can be applied to further elucidate the events occurring in such heated corneas.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

List of Illustrations

Introduction

Although millimeter wave radars are now strategically important, only one study of the effect of millimeter waves on the cornea has been reported (Rosenthal et al, 1975). This study did not use the pulsed mode of millimeter waves which is commonly in use in such radars. Because we have discovered apparent differences between similar doses of pulsed and CW microwaves (Stewart-DeHaan et al, 1980) in preliminary experiments and because we have succeeded in separating the effect of heating from the effects due to the electromagnetic field, for microwaves, we wished to devise a similar system for irradiation of the cornea in vitro which would offer similar advantages for the study of millimeter wave damage to the cornea.

The first step in these experiments was to devise appropriate media and conditions for the tissue culture of corneas, to be used for the experiments investigating their exposure to millimeter waves. The second step, which forms next year's work and is reported in a preliminary way in this report, was to incubate the cultured corneas at different elevated temperatures in order to investigate the effect of incubation at elevated temperature on the cornea.

Materials and Methods

Cell Growth From Cornea

Rabbit eyes are obtained at the nearby slaughterhouse and brought to the laboratory at room temperature for short periods of time (less than half an hour). They are rinsed several times in sterile phosphate-buffered saline (PBS) so as to remove most bacteria. Furthermore, bacterial and fungal growth is inhibited by inclusion of antibiotics (penicillin, streptomycin, and fungagone) in the alpha medium used for the incubations.

Corneas are removed by trimming around the edge of the cornea using iris scissors, and placed right side up on the bottom of a culture dish with alpha medium, pressed down gently by covering with a microscope cover slide loosely attached to the bottom of the dish with dabs of silicone grease at its corners and observed by phase contrast microscopy.

Results

The cell outgrowth from the edge of the corneal explant began to be obvious after only one day of incubation (Figure 1). Growth continued for several days as cells migrated off the edge of the cornea and became attached to the plate.

Phase Contrast and Interference Phase Microscopy

Corneal outgrowth from explants on culture dishes is examined by an inverted microscope (Leitz Diavert) equipped with optics which permit either interference phase or traditional phase contrast microscopy. For convenience, usually the more customary phase contrast optics were used in these studies.

Embedding in Water-Soluble Embedding (WSE) Media, and Thin Sectioning

We have now developed this technique for small pieces of lens and are

presently extending it to cornea. The technique provides for improved resolution of microscopic details (as compared to conventional wax-embedding) because thin (1 μ) sections are cut by glass knives from corneas embedded in plastic formed from water-soluble methacylate. Such sections may be stained by conventional techniques (toluidine blue or hematoxylin and eosin) or subjected to radioautography.

Growth of Whole Corneas

Whole rabbit corneas removed as indicated in the previous section are supported by a triangular frame work (Buck, 1979) so that the alpha medium is in contact with the lower surface of the cornea only, while the upper epithelial surface remains exposed to the air.

The Effect of Elevated Temperature on Corneal Epithelial Cells

A fresh cornea is excised and placed in a beaker containing PBS at specific temperature, tested (37°, 39°, 42°, 45°, 50°) for 30 mins. exactly. The cornea is removed and placed in Karnovsky's fixative at 40°C, then placed in a refrigerator and held overnight (24 hours). The fixative is replaced by 0.2M cacodylate buffer, and the sample processed for SEM as usual (Figure 3).

Cytoskeletal Visualization

1) High Voltage EM

We hope to investigate this autumn whether samples for WSE media may also be suitable directly for high voltage electron microscopy to enable visualization of the cell cytoskeleton.

2) Scanning electron Microscopy

Dr. Creighton has revealed the structure of cytoplasmic matrix and nucleus i.e. the cytoskeleton of corneal cells by removing the overlapping cell membrane adhering to Scotch "Magic" brand tape pressed on the surface of critical-point-dried corneas (Fig. 2).

Biochemical Techniques

Techniques for analysis of protein, glutathione, Vitamin C, malonaldehyde, ATP and adenylate energy charge, lipid analysis and Vitamin E were described in our grant application last year. These have been developed this year for analysis of lens tissue (described in accompanying progress report for Grant No. DAMD-17-80-G-9449). Following the recent development of techniques which appeared to satisfactorily meet the criteria for morphological integrity of corneal cells, we are now investigating the system with respect to these biochemical parameters with a view to optimizing them, and to evaluating the changes, both morphological and biochemical which occur in the cornea when it is subjected to short periods of elevated temperature.

Results

a) Tissue Culture Medium Which Supports Outgrowth of Corneal Epithelial Cells

Several tissue culture media have been tested for ability to support outgrowth of corneal epithelial cells, in a preliminary screening experiment. The culture conditions which appeared to give optimum conditions for corneal maintenance are described in Fig. 1. In these experiments, the corneal epithelial cells migrated and grew from the original explant over a period of several weeks. Although it seems that these conditions are suitable for corneal culture, further study of the relative role of serum in the medium seems necessary since it has recently been suggested by Van Horn's group to result in increased vacuolation of the epithelial cells (Tierney et al, 1981).

b) Cytoskeletal Observation

Although stereoscopic views of cytoskeleton by HVEM are planned for this autumn, a real breakthrough in examination of the cytoskeleton has been made (Fig. 2). Evaluation of cytoskeletal structural changes by a technique involving scanning electron microscopy now seems possible. Although further work will be required to establish definitively how this technique may be applied and interpreted, removal of the cell membrane by simple adhesion to Scotch brand "Magic" tape provides a quick and easy technique for visualizing the internal cell contents.

Discussions: Cytoskeletal Observation

The Scotch tape technique for cytoskeletal visualization has the advantages of permitting the fixed cell contents and nuclear contents of the fixed cell to be observed in a "native" state. Several other techniques which have been used for removal of cytoplasmic membranes involve the solublization of the cell membrane by detergent solutions. Although these techniques have also permitted visualization of cytoplasmic contents, the exposed cytoplasm has also been subjected to the detergents, which may give rise to artefacts and complicate interpretation of the results.

c) Effects of Elevated Temperature on the Corneal Epithelium

Elevated temperatures seem to cause striking changes in morphology of the corneal epithelium (Fig. 3). At 37°, the junctions between corneal epithelial cells appear as a ridge and the cells surfaces are covered by short stubby cylindrical microvilli. At 39°, the junction between cells has become a trough, possibly due to cell swelling and microvilli are disorganized, elongated and intertwined cylinders. At 42°, the epithelial cells have torn apart and some are missing from the surface, leaving devided areas; microvilli are sparse and appear only as small bumps on the surface. After exposure to higher temperatures, (45°C and 50°C) a progression appears to occur involving cells curling up and apparently dying with complete loss of microvillae, resulting in denudation of large areas of such corneas.

Conclusions

These results establish conditions for successfully culturing isolated rabbit corneas, which we proposed as a first stage of our studies of millimeter wave effects on the cornea. Further refinements to the medium may be made in the future in order to optimize certain criteria, both morphological (viz. vacuole formation) and biochemical (ATP glutathione, etc.). In particular several important observations were made:

(1) Corneal Culture

Many aspects of corneal epithelial cell growth can still be maintained in standard tissue culture medium, although the usual control of cell proliferation seems to be partially lost. Further work needs to be done on the effects of serum and additives such as vitamins A and E on the cultured cells.

(2) Cytoskeletal Visualization

The technique using cell membrane removal by Scotch "Margic" tape followed by SEM visualization promises to be an independent means of rapidly investigating cytoskeletal changes. The morphological changes (noted below), occurring in response to elevated temperature in the corneal epithelial cells, are very likely to involve such cytoskeletal alterations, since we and others have shown that changes in cytoskeletal components can cause morphological alterations in cultured cells, such as "arborization" by cytochalasin D. In these previous studies we also indicated a possible link between cytoskeletal structure and ability of the (lens) cells to express a particular biochemical function.

The confirmation and supplementation of these SEM studies by HVEM will be helpful in further elucidating the structure of the cytoplasmic and nuclear matrix. Such studies will provide important information on the normal cytoplasmic matrix which can be used for comparison purposes in planned studies of effects of (1) heat and (2) millimeter wave irradiation on the cornea.

(3) Effects of elevated Temperature on Isolated Cornea

We observed, as temperature of incubation increased, a depression at the junction between epithelial cells and disruption of the normal microvillae structure, progressing at higher temperatures to gross alterations in cell morphology and denudation of the cornea. These experiments made use of a graduated approach to the study of corneal epithelial injuries, unlike many previous studies of corneal injury and wound healing in which complete denudation of small areas of the complete cornea, or severe burns to the cornea are investigated.

Further study of these stages should permit elucidation of the initial events in damage to the corneal epithelial cells.

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Figure 1: Phase contrast microscopy of corneal epithelial cell explants growing in tissue culture medium for varying periods of time.

a) b) Outgrowth one day after explantation. Dark area is the body of the cornea from which the cells are migrating.

c) d) Outgrowth 3 days after explantation. All cells retain the rounded appearance of the migrating corneal epithelia.

e) f) Outgrowth 5 days after explantation. The cells near to the cornea have flattened and attached to the glass giving a paving stone appearance to the monolayer. At the advancing edge of the monolayer rounded cells still predominate.

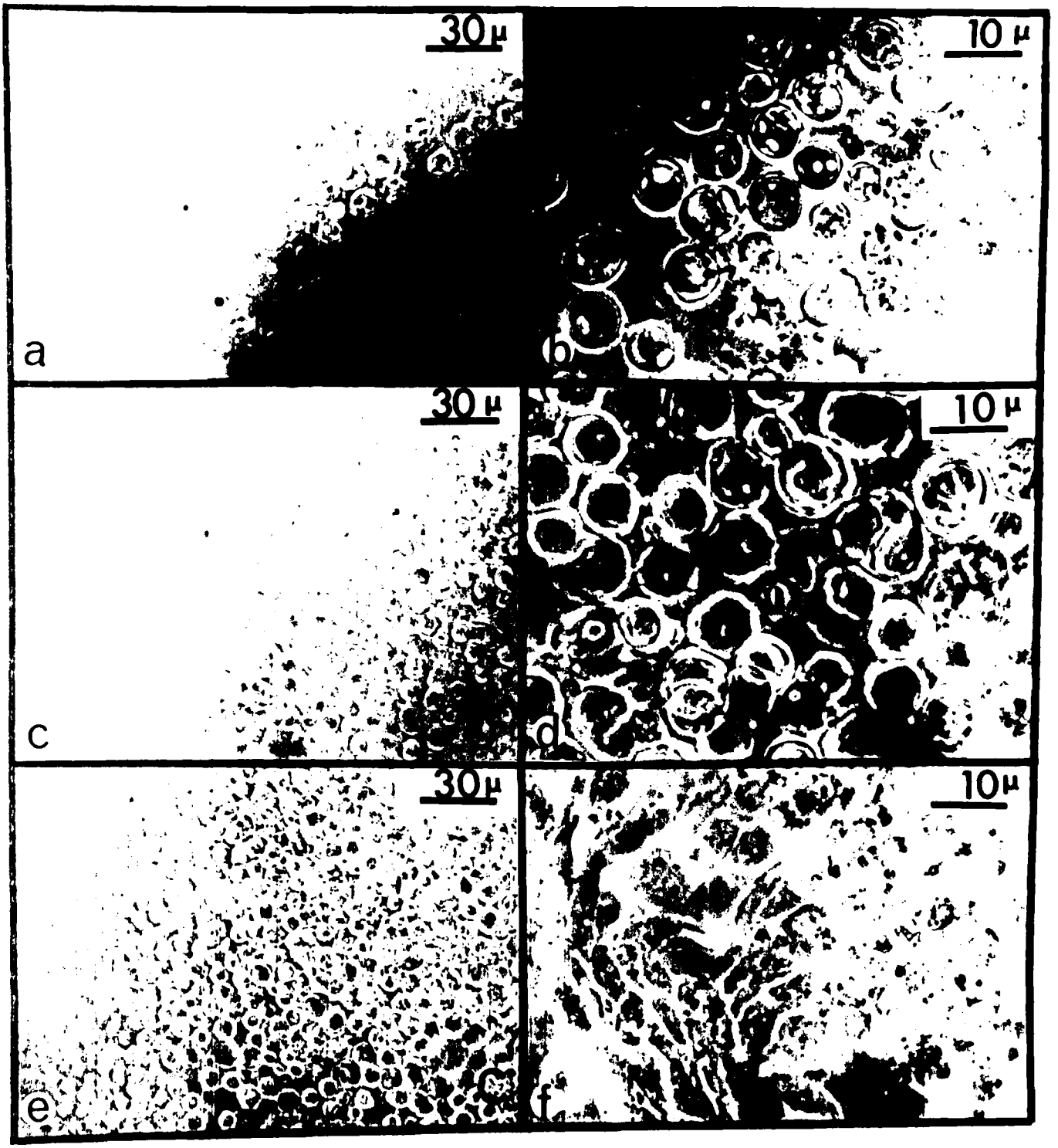


Figure 2: Scanning electron microscopy of epithelial cells of fixed rabbit corneas, treated with adhesive tape to expose the cytoplasmic matrix.

a) A normal group of corneal epithelium cells.

b) A single corneal epithelial cell from which the outer membrane (O) has been pulled away exposing the interior (I). The nucleus (N) and cytoplasmic matrix are visible.

c) d) Cells from which the outer membrane, the nuclei and the cytoplasmic matrix have been removed showing the nuclear pit (NP) and the inside surface of the cell's membrane (I).

e) f) corneal epithelial cells showing various aspects of the cells nuclear membrane (NM), cytoplasmic matrix in the interior of cell (I), the microvilli on the outer surface of the cell (O) and the nuclear interior structure (NI).

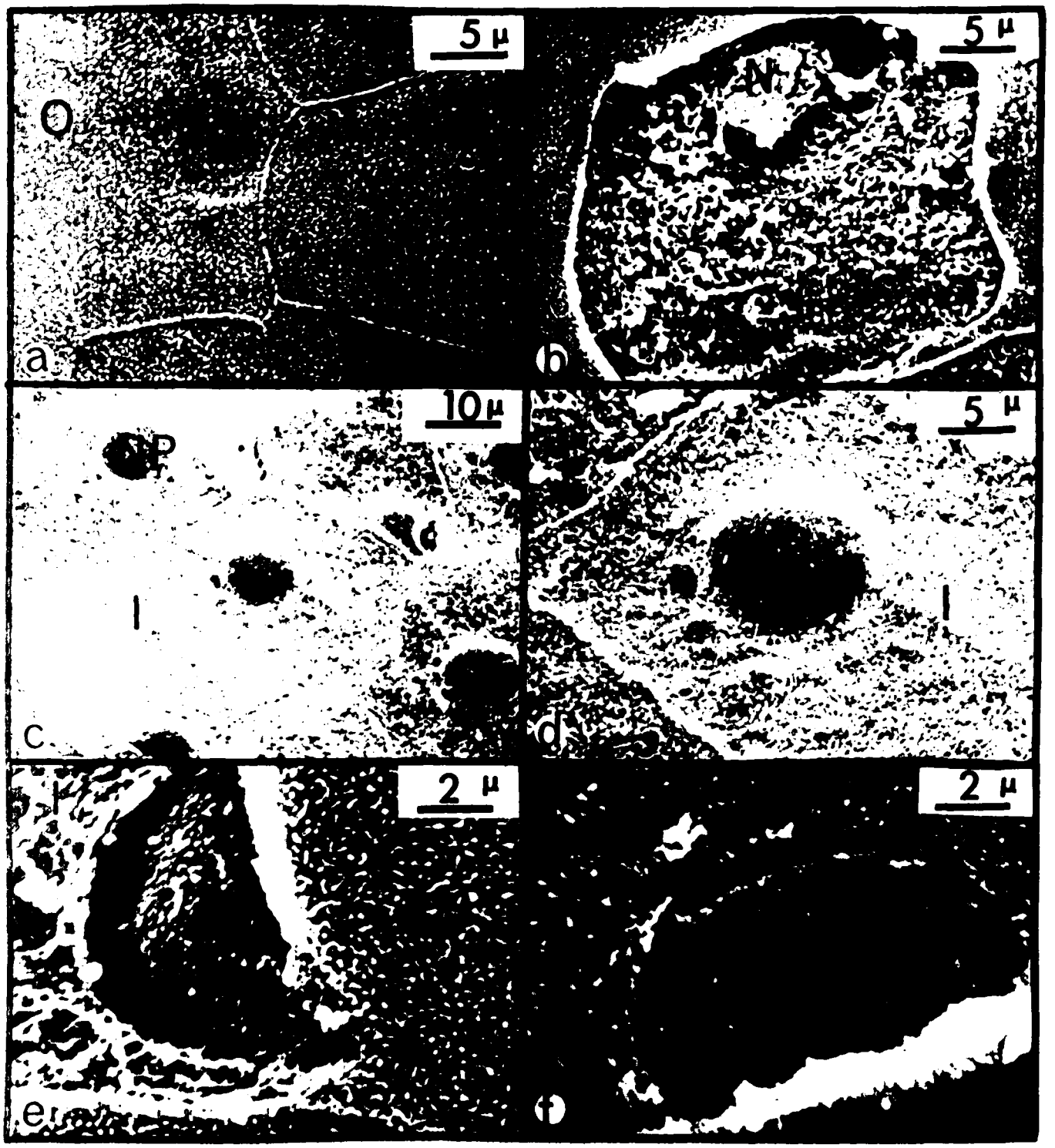


Figure 3: Scanning electron microscopy of epithelial cells of rabbit corneas incubated *in vitro* at various temperatures, illustrating progressive changes in corneal cells as temperature increases. Methods of incubation is described in the text. Temperatures of incubation are indicated on the appropriate photograph.

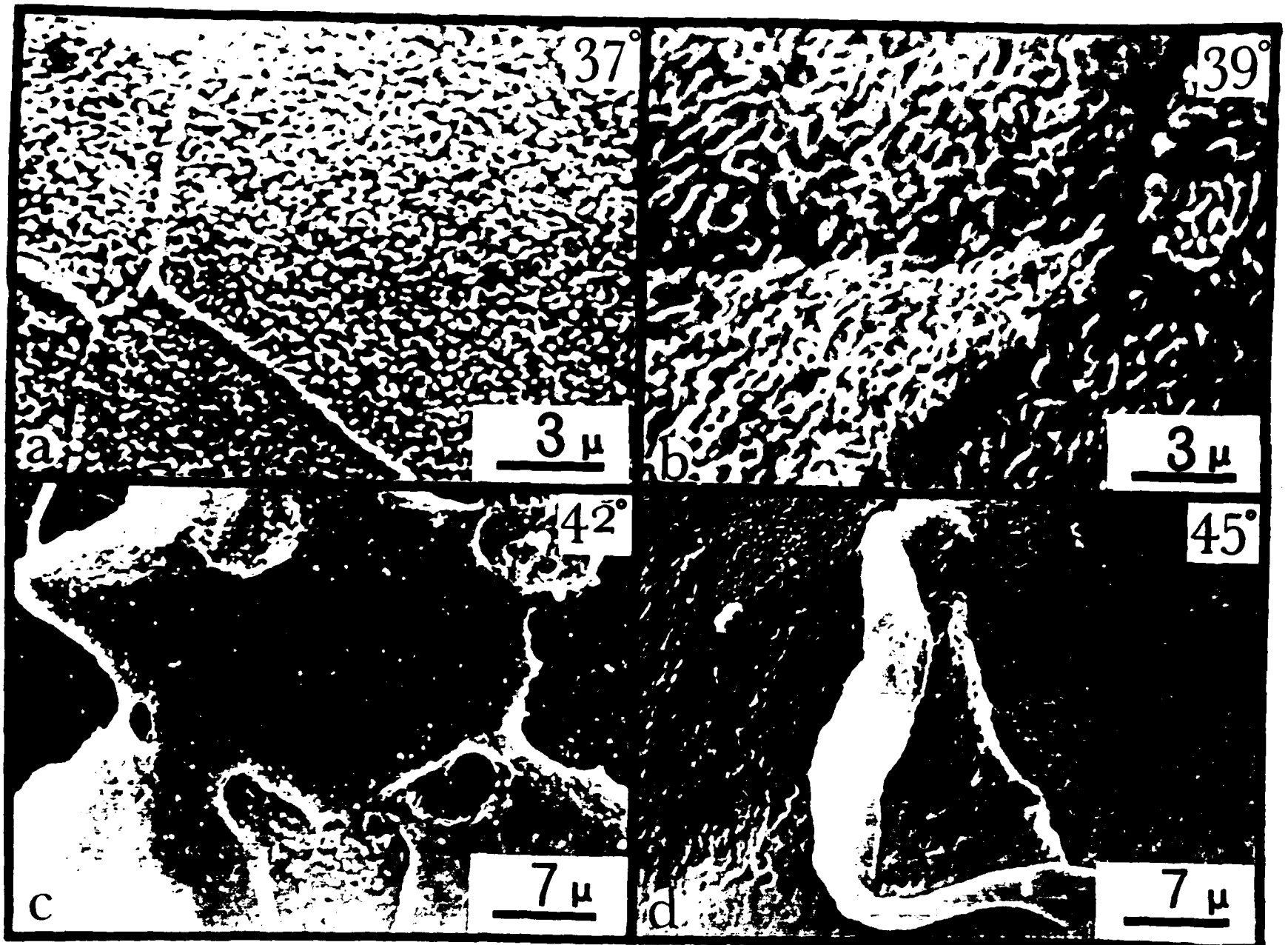
37° - Junction of cells appears as a ridge, microvilli makes a lawn over all of cells, short stubby cylindars.

39° - Junction of cells has become a trough, possibly due to swelling of cells. The microvilli still make a lawn over the cells, but are long intertwined cylindars.

42° - Cells have torn apart. Some are curled up, some missing from surface leaving devided areas. Microvilli are sparse and appear only as small bumps on surface.

45° - Most of cells gone leaving devided surface. Those cells remaining curled up and surface devoid of microvilli.

50° - Most of cells gone but still some areas of curled cells seen.



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